



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|---|
| (51) International Patent Classification ⁵ : A61K 39/04, G01N 33/569 C07K 7/06, 7/08, 13/00 | A1 | (11) International Publication Number: WO 91/14448 (43) International Publication Date: 3 October 1991 (03.10.91) |
| (21) International Application Number: PCT/SE91/00225 (22) International Filing Date: 22 March 1991 (22.03.91) (30) Priority data: 9001105-7 27 March 1990 (27.03.90) SE (71) Applicant: AKTIEBOLAGET ASTRA [SE/SE]; S-151 85 Södertälje (SE). (72) Inventors: CHINNASWAMY, Jagannath ; 41, III Cross, Cubbonpet, Bangalore-560003 (IN). MEENAKSHI, Balganesha ; 304, Shashi Kiran Apartments, 18th Cross, Malleswaram, Bangalore-560055 (IN). BACHALLY, Ramasastri, Srinivasa ; 65, 14th Cross, I Block, R T Nagar, Bangalore-560032 (IN). | | (74) Agents: MIKSCHE, Gerhard et al.; AB Astra, Patent Department, S-151 85 Södertälje (SE). (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent). Published <i>With international search report.</i> |
| (54) Title: NEW PROTEINS, PEPTIDES AND CORRESPONDING DNA OR RNA SEQUENCES AND PROBES USEFUL FOR DIAGNOSING TUBERCULOSIS (57) Abstract <p>Disclosed are the immunochemical properties of a 17 kDa protein antigen fractionated, purified and sequenced for amino acids from <i>Mycobacterium tuberculosis</i> (South Indian isolate, SII 1) which causes human tuberculosis worldwide among 16 million patients. The 17 kDa protein antigen which has an N-terminus of A T T L P V Q R (aa 1-8) has at least three specific antibody binding epitopes located on linear peptides of sequences, R A T Y D K R Y E V R (aa 91-101) and S E F A Y G S F V R (aa 68-77) which were useful in a micro ELISA for the early diagnosis of human tuberculosis by the detection of specific antibodies. The 17 kDa protein antigen which was mitogenic for human tuberculous peripheral blood lymphocytes was found to carry three predicted T-cell epitopes on linear peptides of sequences, S E F A Y G S F V R (aa 68-77) and A E L P G V D P D C D V C I T R (aa 107-122). The 17 kDa antigen of <i>M. tuberculosis</i> (SII 1) which thus had both B and T cell reactive properties was found to contain 131 amino acids and potentially applicable in the immuno diagnosis, immuno therapy and immuno prophylaxis of human tuberculosis.</p> | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria | ES | Spain | MG | Madagascar |
| AU | Australia | FI | Finland | ML | Mali |
| BB | Barbados | FR | France | MN | Mongolia |
| BE | Belgium | GA | Gabon | MR | Mauritania |
| BF | Burkina Faso | GB | United Kingdom | MW | Malawi |
| BG | Bulgaria | GN | Guinea | NL | Netherlands |
| BJ | Benin | GR | Greece | NO | Norway |
| BR | Brazil | HU | Hungary | PL | Poland |
| CA | Canada | IT | Italy | RO | Romania |
| CF | Central African Republic | JP | Japan | SD | Sudan |
| CG | Congo | KP | Democratic People's Republic of Korea | SE | Sweden |
| CH | Switzerland | KR | Republic of Korea | SN | Senegal |
| CI | Côte d'Ivoire | LI | Liechtenstein | SU | Soviet Union |
| CM | Cameroon | LK | Sri Lanka | TD | Chad |
| CS | Czechoslovakia | LU | Luxembourg | TG | Togo |
| DE | Germany | MC | Monaco | US | United States of America |
| DK | Denmark | | | | |

NEW PROTEINS , PEPTIDES AND CORRESPONDING DNA OR RNA SEQUENCES AND PROBES USEFUL FOR DIAGNOSING TUBERCULOSIS.

1. Summary of the invention

Disclosed are the immunochemical properties of a 17 kDa protein antigen fractionated, purified and sequenced for amino acids from Mycobacterium tuberculosis (South Indian isolate, SII 1) which causes human tuberculosis worldwide among 16 million patients. The 17 kDa protein antigen which has an N-terminus of A T T L P V Q R (aa 1-8) has at least three specific antibody binding epitopes located on linear peptides of sequences, R A T Y D K R Y E V R (aa 91-101) and S E F A Y G S F V R (aa 68-77) which were useful in a micro ELISA for the early diagnosis of human tuberculosis by the detection of specific antibodies. The 17 kDa protein antigen which was mitogenic for human tuberculous peripheral blood lymphocytes was found to carry three predicted T-cell epitopes on linear peptides of sequences, S E F A Y G S F V R (aa 68-77) and A E L P G V D P D C D V C I T R (aa 107-122). The 17 kDa antigen of M. tuberculosis (SII 1) which thus had both B and T cell reactive properties was found to contain 131 amino acids and potentially applicable in the immuno diagnosis, immuno therapy and immuno prophylaxis of human tuberculosis.

2. Field of invention

The present invention relates to a novel 17 kDa protein antigen of Mycobacterium tuberculosis (South Indian Isolate SII 1) and certain peptide fragments derived therefrom, and to the use of the said antigen, and of the peptide fragments

derived therefrom, in immuno diagnosis, immunotherapy and immuno prophylaxis of human and experimental tuberculosis. The invention also relates to the DNA sequence coding for the said 17 kDa antigen, to DNA sequences coding for the said peptide fragments derived from the 17 kDa antigen and to the DNA and RNA probes constructed on the basis of the protein sequence of the 17 kDa antigen including the sequences of the peptide fragments of the 17 kDa antigen. A major field of use is the use of 17 kDa antigen and peptide fragments thereof in immuno diagnosis of tuberculosis. A further field of use is the use of the 17 kDa antigen or of the said peptide sub structures thereof for the preparation of a vaccine against tuberculosis. A further field of use is the use of the 17 kDa antigen or its sub structure peptides for the detection of T cell proliferation by skin tests or invitro tests in man. This last mentioned field of use is of importance in the possible treatment of human cancer by the boosting of cellular immunity. A further field of use is the use of the 17 kDa antigen or its sub structure peptides for the laboratory production of cellular growth factors and enzymes.

3. Background of invention

Human tuberculosis caused by M.tuberculosis is an important chronic debilitating disease occurring worldwide affecting about 16 million people. While it is prevalent mostly in under developed and developing countries, the recent epidemic of Acquired Immuno Deficiency Syndrome (AIDS) among developed

countries has posed a problem of secondary infection with mycobacteria including M.tuberculosis.

Precise and confirmative diagnosis of human TB enables early treatment for which powerful antibiotics are available, although in many countries drug resistance has become a problem. Nevertheless, early diagnosis of TB means effective chemotherapy and thus elimination of the transmission of live bacilli from pulmonary TB patients.

Conventional diagnosis of TB depends upon the clinical and radiological findings, microscopy of tuberculous specimen for bacilli, and bacteriological isolation in culture of M.tuberculosis.

Global control of TB has not yet been established because of the inadequacy of the presently available methods of diagnosis.

Thus many clinical features of TB are not specific to TB alone and a study in India (source: National Tuberculosis Institute, Bangalore, India) revealed that only 30% of the X-ray suspects eventually developed TB although, in many countries including India, all X-ray suspects are put on anti tuberculous chemotherapy.

The microscopy of tuberculous specimen is not easy under field conditions and atleast 10^4 bacilli/ml are required for effective screening. Many tuberculous specimens like cerebro spinal fluids from tuberculous meningitis infrequently contain bacilli. Further, bacteriological culture generally

takes 6 to 8 weeks and is expensive as a routine diagnostic measure.

The widely used tuberculin skin test lacks sensitivity and specificity and takes about 3 days for completion. Since chemotherapy of TB requires compliance for atleast 6 months, many patients who are irregular in treatment develop drug resistance and transmit live bacilli. Finally, the traditional BCG vaccination has now been found to give varying levels of protection depending upon geographic regions.

Consequent to these factors, many investigators including the WHO recommend that early diagnosis of TB should be considered as a priority area of research and development.

Mycobacteria are powerful immunogens for man and animals, as evidenced by their use in immuno adjuvants to boost immune responses. Thus, many antigens derived from M.tuberculosis have been known to induce the formation of specific antibodies and proliferative lymphocytes among TB patients (Ivanyi et al., 1988) and experimental animal models. The detection of an antibody response in TB has a potential application in early diagnosis. Likewise, the study of M.tuberculosis specific T lymphocytes has application in early diagnosis by skin tests and development of protective vaccines.

Thus the focus of research in TB pertaining to these aspects lies in the identification and synthesis of M.tuberculosis specific antigens.

SUBSTITUTE SHEET

4. Prior Art

Many investigators have identified protein antigens of M.tuberculosis which have potential immuno diagnostic or immuno prophylatic application. The N-terminal and internal amino acid sequences of many of these antigens have been published. The N-terminal amino acid sequences of some of these are presented in Table 1.

Table I: M.tuberculosis protein antigens identified
by N-terminal amino acid sequences.

| Investigators | Antigens and N-terminus |
|--------------------------------|-----------------------------|
| Shinnick <u>et al</u> , 1987 | 65 kDa: R G C R H P V |
| Yamaguchi <u>et al</u> , 1987 | MPB 57: M A K F N I K P L |
| Pattorroyo <u>et al</u> , 1987 | 13 kDa: A K V N I |
| | 18 kDa: G D L V G P G A E |
| | 23 kDa: A P K T Y |
| | 30 kDa: F S X P G L |
| | 68 kDa: W M T M T |
| | 77 kDa: G K X I A Y D G A A |
| Matsuo <u>et al</u> , 1988 | 30 kDa: F S R P G L P |
| Ashbridge <u>et al</u> , 1989 | 19 kDa: E H R V K R G L T V |
| Baird <u>et al</u> , 1989 | 10 kDa: A K V N I P K P |
| Garcia <u>et al</u> , 1989 | 70 kDa: F Q R I T R Q D L L |
| Borremans <u>et al</u> , 1989 | 32 kDa: F S R P G L P |

To our knowledge, none of these antigens have been introduced as immuno diagnostic test products. Two of these antigens (10 kDa of Baird et al, 1989 and a homologue of 65 kDa

SUBSTITUTE SHEET

antigen of Shinnick et al, 1987) have been tested for vaccine potency among experimental animal models where they showed poor protection against M.tuberculosis (D.W. Smith, University of Wisconsin, USA, personal communication).

An ELISA kit for diagnosing TB using A60 antigen present in all mycobacteria has been introduced by ANDA diagnostics (France). The test is thus not specific for human TB alone.

An alternative method of diagnosis of TB has been the use of DNA probes. The commercially available GEN PROBE kit (1988) is used to confirm the identity of members of M.tuberculosis complex isolated in culture and has not been used directly on clinical specimen. A positive result with this test does not rule out other mycobacteria. The DNA probe devised by Enzo Biochem (J.Clin Microbiol.1988, Dec) used specific DNA sequences of 1000 bases or more in length and claimed to be more specific. A major handicap of all DNA based probes is that whether a true positive reaction can be obtained with specimens of patients so as to avoid the laborious culture of bacilli.

Phenotypic variation in virulence is known among the M.tuberculosis strains isolated in South India (Naganathan et al 1987), the molecular basis for which is not yet established. Abou Zeid et al, (1988) found that a 13 kDa protein antigen was present in phage type II virulent M.tuberculosis and absent in phage type I South Indian low virulent M.tuberculosis. However, the relationship between this antigen and virulence is yet to be investigated in detail.

SUBSTITUTE SHEET

5. The Invention :

A 17 kDa protein antigen was found to be present among the isolates of M.tuberculosis. The immuno chemical features of this antigen are disclosed under this invention.

Thus the present invention relates to:

1. The 17 kDa protein antigen of M.tuberculosis (SII 1) as defined below and certain sub structures (peptides) of 17 kDa protein antigen as defined below.
2. A DNA sequence coding for the 17 kDa antigen from M.tuberculosis (SII 1).
3. A DNA sequence coding for the sub structures (peptides) of the 17 kDa antigen from M.tuberculosis (SII 1).
4. The use of the said 17 kDa protein or the said sub structures (peptides) for the preparation of monoclonal or polyclonal antisera which react with the said 17 kDa protein antigen or sub structures thereof. The said antibodies can be raised in mammals like mice, rabbits and goats for polyclonals and in mice for monoclonals.
5. The use of the said 17 kDa protein antigen of the said sub structures (peptides) for the detection of antibodies among human and animal specimen for immuno-diagnosis. Detection methods are known in the art one employs such as ELISA, radio immuno assay (RIA) and reverse passive hemagglutination (RPHA).

SUBSTITUTE SHEET

6. The use of the 17 kDa protein antigen or sub structures (peptides) thereof for the treatment of tuberculosis.

7. The use of the 17 kDa protein antigen or substructures peptides thereof for the preparation of a vaccine against tuberculosis.

8. The use of the 17 kDa antigen or sub structures (peptides) thereof for the preparation of a reagent for the skin test in immuno diagnosis for tuberculosis.

9. DNA or RNA probes constructed on the basis of the protein sequence of the 17 kDa antigen or sub structures (peptides) thereof for diagnosis of tuberculosis. Such probes can be constructed by methods known in the art. Labelling of such probes can be done by known methods such as radioisotope incorporation or by non radio-active labelling use for example, biotin.

10. A method of diagnosis of human tuberculosis by interacting body fluids such as serum, CSF, pleural fluids from a patient to be diagnosed with a monoclonal antibody to the 17 kDa antigen or its sub structures (peptides) as defined in paragraph 3 above.

11. A method for diagnosis of human tuberculosis by interacting a body fluid such as serum from a patient to be diagnosed with a 17 kDa protein as defined in paragraph 5 above.

12. A method of diagnosing human tuberculosis by interacting body fluids such as sputum, serum, CSF and pleural fluids

from a patient to be diagnosed with a DNA or RNA probe as defined in paragraph 9 above.

13. A method for in vitro detection of human tuberculosis which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody as defined in paragraph 10 in labelled form.

14. A method for in vitro detection of human tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody as defined in paragraph 10 in labelled form.

15. A method for in vitro detection of human tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a polyclonal antibody as defined in paragraph 3 in labelled form.

16. A kit for performing immuno diagnosis of tuberculosis utilizing a monoclonal antibody to 17 kDa antigen as disclosed in paragraph 3 above.

17. A kit for immuno diagnosis of tuberculosis utilizing 17 kDa antigen or peptide sub structures as disclosed in paragraph 5 above.

18. A kit for diagnosing tuberculosis utilizing a DNA or RNA probes as disclosed in paragraph 9 above.

19. A micro organism expressing a 17 kDa protein or sub structures thereof as disclosed in paragraph 1 above.

20. A vaccine against tuberculosis developed on the basis of the 17 kDa antigen or sub structure peptides thereof as disclosed in paragraph 1 above. Such a vaccine can be a product of genetically engineered organisms such as Salmonella, Vaccinia virus etc.

The present invention is exemplified by but not limited to the diagnosis, therapy or prophylaxis of diseases, especially diagnosis of M.tuberculosis infection. Epidemiological screening, forensic investigations, determination of food contaminations, public health surveys, preventive medicine, veterinary and agricultural applications with regard to the diagnosis of infectious agents may be covered by this disclosure.

5.1 Fractionation and purification of 17 kDa antigen.

5.1.1 Crude sonicate antigen.

M.tuberculosis (SII 1) was cultured at 37 ° C for 2 weeks in

Kirchner's medium and harvested bacilli were killed in cold acetone for 18 h at 4 ° C. The bacilli were washed thrice with saline and a suspension of 10 mg bacilli in 5 ml saline was sonicated at 40 watts output using a miniprobe of a Branson sonifier. The sonicate was centrifuged at 20,000 x g for 30 min and the supernatant was estimated for protein content (Lowry's method) prior to freeze storage at -70 ° C.

5.1.2 Fractionation and purification.

500 ug of crude sonicate was fractionated on 12.5% sodium dodecyl sulfate polyacrylamide gel as described by

Hunkapiller and Lujan (1986). The protein bands were visualized by brief staining with coomassie brilliant blue and the 17 kDa antigen was electroeluted against 0.05 M ammonium bicarbonate with 0.1% SDS followed by electro-dialysis against 0.01 M ammonium bicarbonate with 0.02 % SDS. The eluted protein was then extracted with chloroform-methanol to remove SDS and the precipitate was dried.

The purity of the eluted protein was analysed by subjecting 5 ug of this precipitate to HPLC using Lichrosorb RP 18 column (LKB). A single peak eluted at 45% B and 26 min was found to contain immuno reactive antigen. Fig. 1 shows the HPLC profile of 17 kDa antigen.

5.2 Amino acid sequence analysis of 17 kDa antigen

5.2.1 Peptide mapping of 17 kDa antigen

Tryptic map: 30 ug of 17 kDa protein was digested with TPCK treated trypsin with an enzyme to substrate ratio of 1:50 in 0.1 M ammonium bicarbonate buffer, pH 7.8 at 37 ° C for 5 h. The tryptic digest was fractionated by HPLC on RP 18 column (0.46 x 25 cm) equilibrated with solvent A (0.1% TFA in water) and the peptides were eluted with a gradient of solvent B (70% acetonitrile containing 0.085% TFA) from 0 to 65% in 60 min. The tryptic map is shown in Fig. 2.

V8 protease map: 30 ug of 17 kDa antigen was treated with staphylococcal V8 protease for 48 h in 0.07% ammonia at 37 ° C. The molar ratio of the enzyme to substrate was 1:25. The various peptides in the enzyme digest were purified on an HPLC column under the conditions used for tryptic mapping. The peptide profile is shown in Fig. 3.

5.2.2 Sequence analysis of 17 kDa antigen

The amino acid sequence analysis of the protein and the peptide was done using protein sequencer model 477A (Applied Biosystems Inc., USA) with an on line PTH amino acid analyser. The sample was solubilized in 10% formic acid and fixed onto a polybrene coated (1 mg) TFA treated glass fibre disc and used for sequencing.

The first 18 amino acids from the N-terminal was determined using the whole protein. Based on the amino acid sequences of the tryptic peptides, V8 protease was selected to generate the peptides that could give the overlaps for the tryptic peptides. The alignment of both tryptic and V8 protease peptides gave the complete sequence for the 17 kDa antigen. The details of the overlaps are given in Fig. 4.

5.2.3 Amino acid composition of 17 kDa antigen

The protein has A9, C3, D11, E10, F9, G8, H2, 17, K4, L11, M2, P9, Q2, R12, S8, T9, V11 and Y4. It is significant in not having tryptophan and asparagine. The protein is acidic in nature since it has 5 acidic amino acids (D+E=21) in excess of the total number of basic amino acids (R+K=16). The protein has 131 amino acids that account for a molecular weight of 14,762.

5.3 Immuno reactivity of 17 kDa antigen

5.3.1 Demonstration that 17 kDa antigen is immunodominant in M.tuberculosis strains.

M.tuberculosis (SII 1), M.tuberculosis ATCC 27294, M.phlei, M.

smegmatis, M.kansasii, M.avium intracellulare and M.scrofulaceum were cultured in Kirchner's medium for 2 weeks and harvested bacilli were killed in cold acetone. Sonicate antigens were prepared from each of these species as described in section 5.1.1. SDS PAGE analysis of these antigens was then carried out in 12.5% gel. Coomassie blue stained gels showed that the 17 kDa antigen was present only in M.tuberculosis strains. A rabbit antiserum raised to M.tuberculosis SII 1 was also used to probe these sonicates in Western blotting. The dominant 17 kDa band was found in the Western blot.

5.3.2 Demonstration that 17 kDa elicits antibodies among experimental animals.

Electro eluted 17 kDa antigen (10 ug in 100 ul saline) was emulsified with an equal volume of Freund's incomplete adjuvant (FICA) and used for intra peritoneal immunization of 10 BALB/c mice. Serum collected from these mice 30 days after immunization recognized a 17 kDa band in the sonicate antigen of M.tuberculosis SII 1.

Thus this experiment confirms that a polyclonal or monoclonal antibody can be produced in the mouse which recognize the protein structure of 17 kDa antigen or sub structures (peptides) thereof. Such antibodies, in particular the monoclonal antibodies can be used in an antigen detection method like the sandwich ELISA for the detection of the 17 kDa antigen or sub structures thereof among human tuberculosis specimen leading to immuno diagnosis of tuberculosis.

SUBSTITUTE SHEET

5.3.3 Demonstration that 17 kDa antigen reacts with human TB patient sera.

Sera derived from 24 healthy persons and 20 culture proven TB patients were titrated against the 17 kDa antigen as follows. PVC Dynatech plates were coated with 1 ug/ml PBS of electro eluted 17 kDa antigen for 24 h at 22 ° C. PBS-BSA blocked plates were then titrated against duplicate (1/200) dilutions of sera which were incubated at 22 ° C for 2.5 h. Washed plates received anti human IgG HRP conjugate for 1.5 h. Washed plates were then assayed with O-phenylene diamine substrate and read at 492 nm. Table II shows that the 17 kDa antigen had a sensitivity of 70% and specificity of 85%

Table II. Micro ELISA with 17 kDa antigen on sera from TB patients and controls

| Serum groups | n | ELISA + | ELISA- | Sensitivity | Specifici |
|----------------|----|---------|--------|-------------|-----------|
| Healthy people | 24 | 4 | 20 | -- | 85% |
| TB patients | 20 | 14 | 6 | 70% | -- |

Sensitivity: Known positivity among TB patients

Specificity: Known negativity among healthy controls

ELISA + : OD 492 nm ≥ 0.3 at 1/200 dilution (=mean + 2SD of OD 492 nm for healthy controls, n=24).

Thus this experiment confirms that the 17 kDa antigen from M.tuberculosis strains can be used in a micro ELISA system for the immuno diagnosis of tuberculosis in man.

5.3.4 Demonstration that the 17 kDa protein antigen has defined antibody epitopes

The peptide fragments derived from the 17 kDa antigen by tryptic digestion (section 5.2.1, Fig.2) were individually

SUBSTITUTE SHEET

titrated against sera from healthy persons and TB patients as in section 5.3.3. Of the 14 peptides tested, peptides with the sequences RATYDK, YEVR, LEDEMK, LMR, DFDGR and SEFAYGSFVR showed antibody binding activity with sensitivity levels between 17 to 36%. To determine whether these peptides formed linear or conformational antibody epitopes, an inhibition of ELISA was carried out in which each of the peptide was assayed against the other five using the mouse antiserum to 17 kDa antibody. The peptides YEVR and ATYDK were mutually inhibitive thus indicating that they were a part of a complete antibody epitope, which was confirmed also by the determination of the complete structure of the 17 kDa antigen as in section 5.2.2, Fig 4. The other four peptides were linear and probably conformational in the presentation of the antibody epitope. Of the said six antibody epitope bearing peptides, the peptides of the following amino acid sequences were synthesized by solid phase method of Merrifield and were found to contain specific and sensitive antibody binding activities :

RATYDKRYEVR : Sensitivity 65%; Specificity 95%

SEFAYGSFVR : Sensitivity 66%; Specificity 95%

The antibody epitope mapping as described has thus indicated that defined sub structures or peptides of 17 kDa antigen can be synthesized and used for the immunodiagnosis of human tuberculosis in micro ELISA.

The peptide of the sequence RATYDKRYEVRDFDGRAEL was synthesized and found to have a sensitivity of 86.6% and a specificity of 100% when tested on CSF samples from culture proven TBM patients and controls. This peptide is accordingly particularly suitable for use in place of the 17 kDa antigen in the ELISA test.

5.3.5 Demonstration that 17 kDa antigen is lympho proliferative

Peripheral blood lymphocytes (PBL) from healthy donors and TB patients were fractionated and 2×10^6 cells were cultured in presence or absence (control) of 1 ug of 17 kDa antigen for 3 days in RPMI 1640 medium with 10% autologous serum. 24 h before harvesting cultures were pulsed with 1 uCi of 3H thymidine. Table III shows that the 17 kDa antigen was lympho proliferative to the lymphocytes of TB patients (data shown for 2 persons only).

Table III. Lympho proliferative assay with 17 kDa antigen.

| Source (PBL) | 3H thymidine incorporation (cpm, mean of triplicate cultures) | |
|--------------|---|---------------|
| Healthy | Control : 90 | Antigen : 121 |
| TB patient | Control : 110 | Antigen : 650 |

In addition to the lympho proliferative property of the 17 kDa antigen, a method of prediction of T cell stimulatory epitopes (Rothbard and Taylor, 1988) was used to map the probable sites in the structure of the 17 kDa antigen. These T cell epitopes were located on peptides of the following sequences:

SEFAYGSFVR

AELPGVDPDCDVCITR

Thus this experiment indicates that the 17 kDa antigen or sub structures (peptides) can be used to stimulate human peripheral blood lymphocytes. Since stimulated lymphocytes

elaborate several cellular growth and differentiation factors which contribute to the vaccine effect of 17 kDa antigen or its sub structures, the 17 kDa antigen can be used at the first instance as a vaccine against TB and also for non-specifically boosting cellular immunity.

Figure 6 shows the primary structure of the 17 kDa antigen from *M.tuberculosis* containing the biologically active regions, although similar activity need not be ruled out in the unmarked regions.

6. Discussion and summary of test results

The present invention describes the immunochemical properties of a novel 17 kDa protein antigen from *M.tuberculosis* (SII 1 strain). *M.tuberculosis* causes tuberculosis worldwide among 16 million people. Because of inadequacy of the diagnostic procedures available now the disease has not yet been eradicated. The focus of research in recent years has been the development of immuno diagnostic methods for detecting TB at an early stage as well identification of suitable candidates for vaccination since the traditional BCG vaccine has given only a partial protection against TB.

The studies described in this invention show that a novel 17 kDa antigen derived from *M.tuberculosis* has antigenic activity. Its antigenic nature and chemistry were investigated. The biologically active proteins of the protein antigen were mapped and immuno diagnostic methods were developed for the early detection of TB.

Thus the 17 kDa protein antigen which had 131 amino acids was found to contain two peptides of sequence RATYDKRYEVR and

SUBSTITUTE SHEET

SEFAYGSFVR which carried antibody binding epitopes for diagnosis of TB. Further, it contained two peptides which carried predicted T cell stimulating regions in sequences, SEFAYGSFVR and AELPGVDPDCDVCITR. The latter two peptides presumably contributed to the T cell stimulating property of the whole 17 kDa antigen described in this invention. The T cell stimulating property of the 17 kDa antigen and its sub structure peptides means that they could be used in therapy and vaccination for TB.

7. Figure legends.

Fig. 1. HPLC analysis of the electro eluted 17 kDa protein antigen from *M. tuberculosis* (SII 1).

HPLC conditions: RP 18 column (LKB, 10 μ m pore size), A: 0.1% TFA in water, B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% B in 40 min, Sensitivity: 0.08, 220 nm.

Fig. 2. Tryptic peptides of 17 kDa protein antigen from *M. tuberculosis* (SII 1) fractionated by HPLC on RP 18 (LKB, 10 μ m pore size).

Fractionation: A: 0.1% TFA in water, B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% B in 60 min, Sensitivity: 0.08, 220 nm.

Amino acid sequence: Sequence determined are drawn against each peptide.

Fig. 3. V8 protease peptides of 17 kDa protein antigen from *M. tuberculosis* (SII 1) fractionated by HPLC on RP 18 (LKB, pore size 10 μ m).

Fractionation: A: 0.1% TFA in water; B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% in 60 min, Sensitivity: 0.082, 220 nm.

Fig. 4. The primary structure of the 17 kDa antigen of *M. tuberculosis* (SII 1) showing the alignment of peptides. Trp: Trypsin, V8: *Staphylococcus aureus* V8 protease. Superscript

SUBSTITUTE SHEET

arrows denote the amino acid sequence obtained with the whole protein.

(Single letter code used for amino acids).

Fig. 5. The primary structure of the 17kDa antigen from *M.tuberculosis* (SII 1) showing the biologically active regions.

AA 68 to 77: Antibody and T cell epitopes present.

AA 91 to 101: Antibody epitope present.

AA 107 to 122: Two T cell epitopes present.

8. References

1. A.Hopps and E.J. Woods, *Proc.Natl.Acad.Sciences*, (1981) 78,3429
2. Hunkapiller, M,W and Lujan E, In, *Methods in Protein Microcharacterization*, Ed.J.E. Shiveley, Humana Press (1986) pp.89
3. Naganathan, N, etal. *Tubercle* (1986) 67,261
4. Shinnick,F, et al, *J.Bacteriol*, (1987) 169,1080
5. Yamaguchi, Y, etal, *FEBS Letters*, (1987) 240,115
6. Pattorroyo G, et al, *Modern Biotechnology in Health*, Academic press, (1987), pp.110.
7. Ivanyi, J et al, *Springers Seminars Immunopathol*, (1988)10 279
8. Abou-Zeid etal, *J.Gen Microbiol* (1988) 134,531
9. Matsuo M etal, *J.Bacteriol*, (1988) 170, 3847
10. Rothbard, J,B and Taylor, W, *EMBO J* (1988) 7,93
11. Ashbridge S, etal, *Nucleic Acids Res* (1989) 17,1249
12. Boremans et al, *Infection and Immunity*, (1989) 57, 3123
13. Garcia E, etal (1989) *Infection and Immunity* (1989),57,204
14. Baird et al, *J.Gen Microbiol*, (1989)135, 931.

SUBSTITUTE SHEET

CLAIMS

1. The protein of the structure:

1 40
ATTLPVQRHPRSLFPEFSELFAPPSFAGLRPTFDTRELM
80
RSIQITIKLEDEMKGIIYLPVAKHGELRSEFAYGSFVRTVS
131
LPVGADEDDIRATYDKRYEVRDFDGRAELPGVDPDCDVCITRGILTVSVCV

2. Peptides of the structure:

RATYDKRYEVRDFDGRAEL

AELPGVDPDCDVCITR

and fragments of the protein according to claim 1 which include one or more of these peptide sequences.

3. A DNA sequence coding for the protein defined in claim 1.
4. A DNA sequence coding for peptides defined in claim 2.
5. A DNA or RNA hybridization probe, constructed on the basis of the proteins or peptides defined in claim 1 or 2 or on the basis of DNA sequences according to claim 3 or 4, said probe being optionally labelled.
6. An antibody to a protein or peptide as defined in claim 1 or 2.
7. An antibody according to claim 6 which is a polyclonal or a monoclonal antibody, said antibody being optionally labelled.
8. A kit for performing immuno diagnosis of tuberculosis including a protein or peptide according to claim 1 or 2.

SUBSTITUTE SHEET

9. A kit for performing immuno diagnosis of tuberculosis including a monoclonal antibody according to claim 7.
10. A kit for performing DNA based diagnosis of tuberculosis including a hybridization probe according to claim 5.
11. A microorganism capable of expressing a protein or peptide according to claim 1 or 2.
12. A vaccine against tuberculosis including a protein or peptide according to claim 1 or 2.
13. A method of diagnosis of human tuberculosis which comprises interacting body fluids such as serum from a patient with a protein or peptide according to claim 1 or 2.
14. A method of diagnosis of human tuberculosis which comprises interacting body fluids such as sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody according to claim 7 in labelled form.
15. A method of diagnosis of human tuberculosis which comprises interacting body fluids such as sputum, CSF, pleural fluid or serum from a patient with a DNA probe according to claim 5 in labelled form.
16. An in vitro method for detection of human tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a polyclonal antibody according to claim 7 in labelled form.
17. An in vitro method for detection of human

tuberculosis, which comprises contacting a sample of a body fluid such as sputum, CSF, pleural fluid or serum from a patient with a monoclonal antibody according to claim 7 in labelled form.

18. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body in immuno-diagnosis, therapy or vaccination in relation to human tuberculosis.

19. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body for producing mammalian polyclonal antibodies or monoclonal antibodies in mice for use in an immunodiagnostic method like sandwich ELISA which detects the protein according to claim 1 among human tuberculous specimens.

20. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body for the detection of antibodies among tuberculous specimens by serological methods for early detection of tuberculosis.

21. A protein or peptide according to claim 1 or 2 for use in a method of treatment of the human or animal body for the detection of T cell proliferation in a tuberculous specimen for obtaining immuno diagnosis through skin tests or obtaining a candidate for vaccine against tuberculosis.

22. Use of a protein according to claim 1 or 2 for immuno-diagnosis, therapy and vaccination in relation to human tuberculosis.

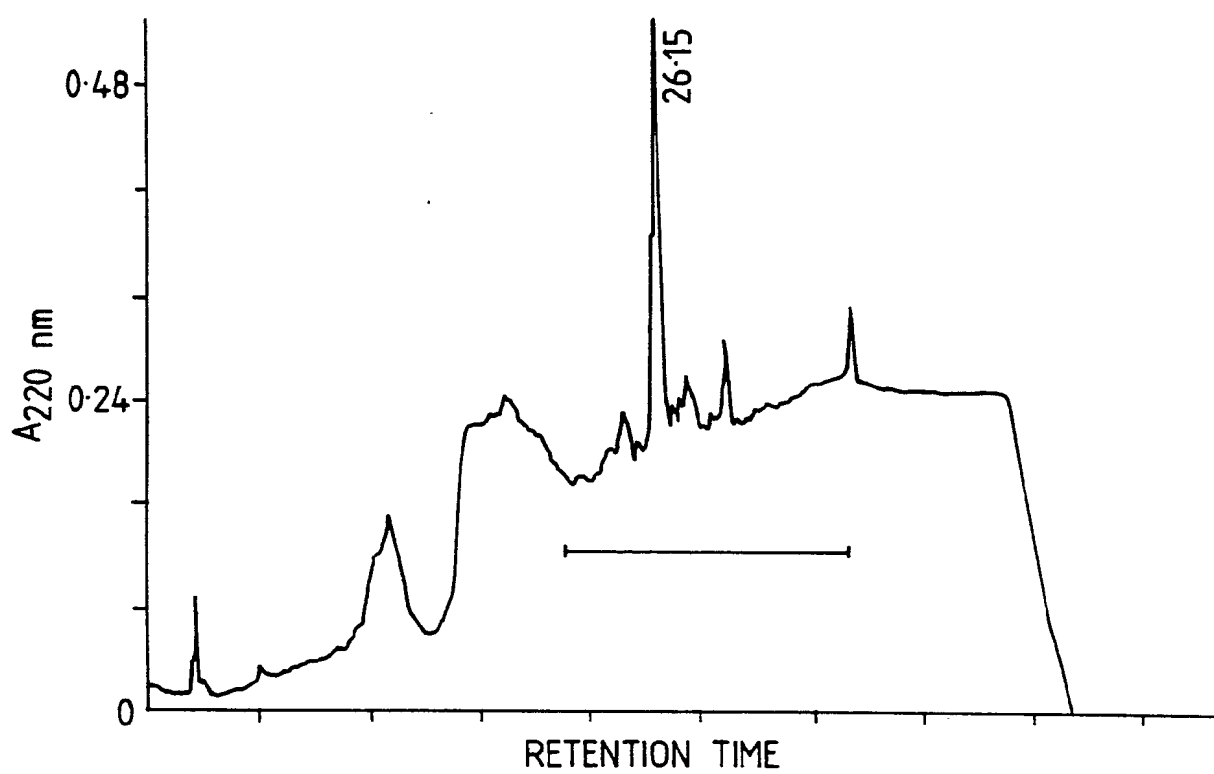
SUBSTITUTE SHEET

23. Use of a protein or peptide according to claim 1 or 2 for developing a reagent for therapy of tuberculous inflammation by T cell proliferation.

24. Use of a protein or peptide according to claim 1 or 2 for the production of growth and differentiation factors using T cell proliferation assay.

25. The use of DNA or RNA probes according to claim 5 for detection of M.tuberculosis DNA among human tuberculous specimen for early detection of tuberculosis.

26. The use of DNA or RNA probes according to claim 5 for the purposes of identification of mycobacterial DNA for example among cultural isolates and for laboratory research.

Fig. 1.**SUBSTITUTE SHEET**

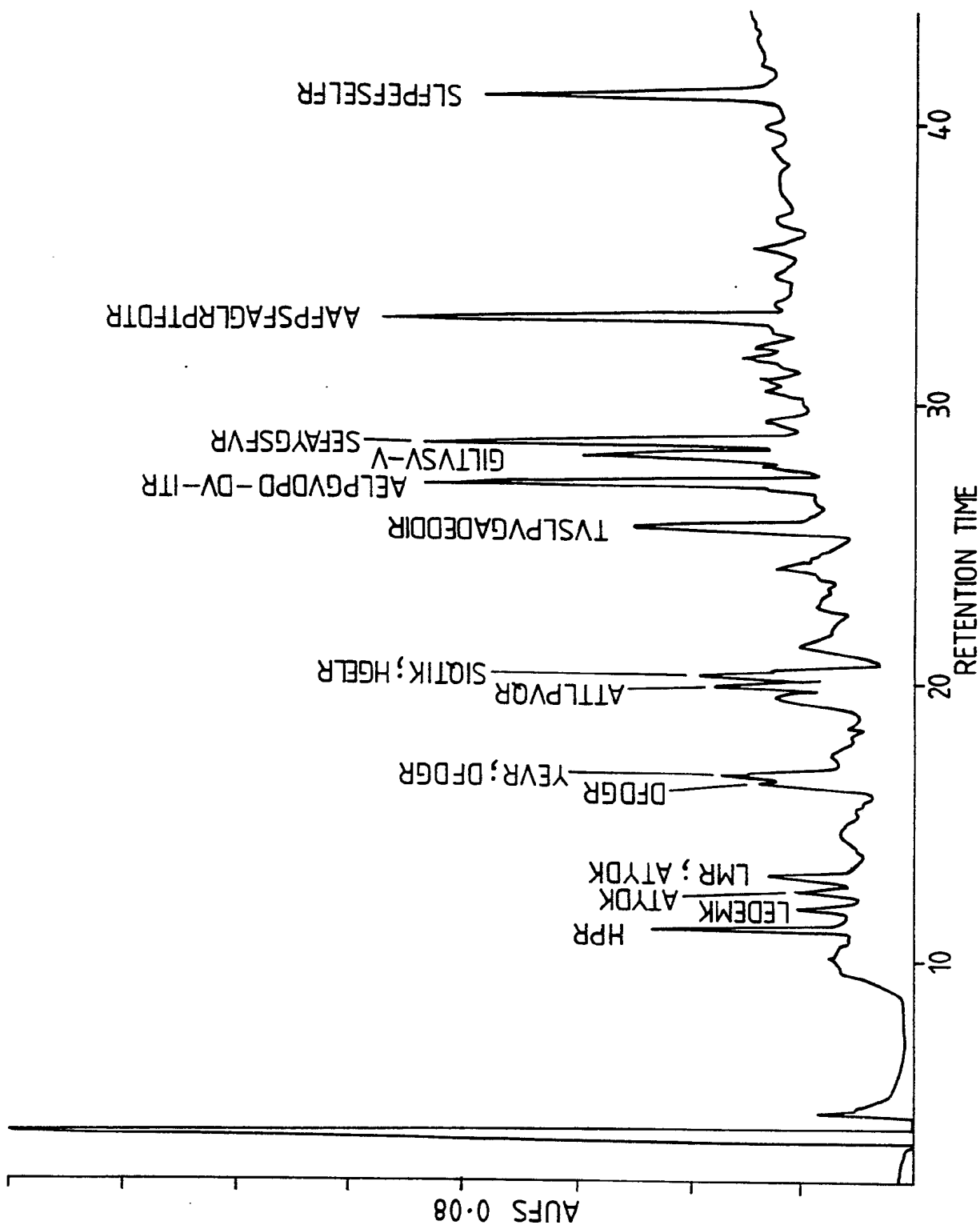
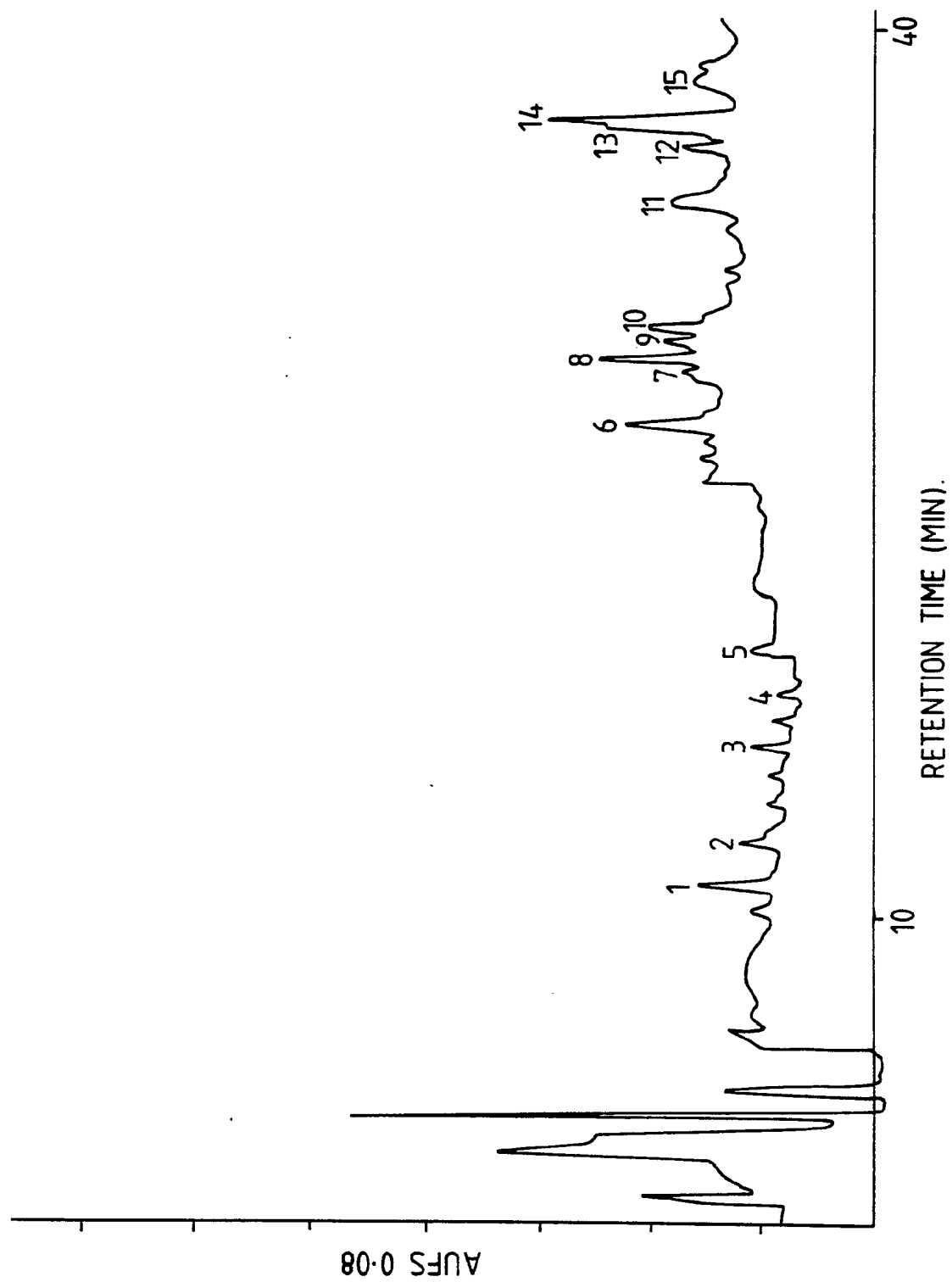


Fig. 2.

Fig. 3.



SUBSTITUTE SHEET

Fig. 4.

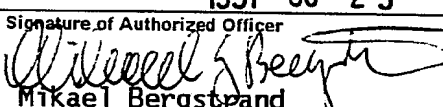
20
 A T T L P V Q R H P R S L F P E F S E L F A
 Trp
 V8
 30
 A F P S F A G L R P T F D T R E L M R S I Q
 Trp
 V8
 40
 I T I K L E D E M K G I Y L P V A K H G E L
 Trp
 V8
 50
 R S E F A Y G S F V R T V S L P V G A D E D
 Trp
 V8
 60
 D I R A T Y D K R Y E V R D F D G R A E L P
 Trp
 V8
 70
 G V D P D C D V C I T R G I L T V S V C V
 Trp
 V8
 80
 90
 100
 110
 120
 130

Fig.5.

⁵ A T T L ¹⁰ P V Q R H P R S L F P E F S E L F A A F P S ²⁵
³⁰ F A G L R P T F D T R E L M R S I Q I T I K L E D E M ⁵⁰
⁵⁵ K G I Y L P V A K H G E L R ⁶⁵ S E F A Y G S F V R ⁷⁵ T V
⁸⁰ S L P V G A D E D D I ⁹⁰ R A T Y D K R Y E V R ¹⁰⁰ D F D G R ¹⁰⁵
¹¹⁰ A E L P G V D P D C D V C I T R ¹²⁰ G I L T V S V C V ¹³⁰

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00225

| | | |
|---|--|-------------------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: A 61 K 39/04, G 01 N 33/569, C 07 K 7/06, 7/08, 13/00 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁷ | | |
| Classification System | Classification Symbols | |
| IPC5 | A 61 K; G 01 N; C 07 K | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸ | | |
| SE,DK,FI,NO classes as above | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ | | |
| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| A | WO, A1, 8806591 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 7 September 1988, see the whole document -- | 1-26 |
| A | WO, A3, 8805823 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 11 August 1988, see the whole document -- | 1-26 |
| A | Journal of Clinical Microbiology, Vol. 26, No. 12, December 1988 Anne Worsaae et al.: "Monoclonal Antibodies Produced in BALB.B10 Mice Define New Antigenic Determinants in Culture Filtrate Preparations of Mycobacterium tuberculosis", see page 2608 - page 2614 -- | 1-26 |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report | |
| 14th June 1991 | 1991 -06- 2 5 | |
| International Searching Authority | Signature of Authorized Officer | |
| SWEDISH PATENT OFFICE |  Mikael Bergstrand | |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|--|----------------------|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| A | Infection and Immunity, Vol. 55, No. 12, December 1987 Anne Worsaae et al.: "Allergenic and Blastogenic Reactivity of Three Antigens from Mycobacterium tuberculosis in Sensitized Guinea Pigs", see page 2922 - page 2927 -- | 1-26 |
| A | Journal of Immunology, Vol. 138, No. 3, February 1987 Fredrik Oftung et al.: "Human Oftung et al.", see page 927 - page 931 -- | 1-26 |
| A | Journal of Clinical Microbiology, Vol. 24, No. 1, July 1986 S.R. Coates et al.: "Identification fo Mycobacterium tuberculosis Antigens in Seibert Fractions by Immunoblotting", see page 126 - page 130 -- ----- | 1-26 |

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00225

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-04-30
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO-A1- 8806591 | 88-09-07 | AU-D- 1495688 | 88-09-26 |
| | | EP-A- 0305488 | 89-03-08 |
| | | US-A- 4952395 | 90-08-28 |
| | | US-A- 4976958 | 90-12-11 |
| | | ZA-A- 8801344 | 88-08-26 |
| WO-A3- 8805823 | 88-08-11 | AU-D- 1548388 | 88-08-24 |
| | | EP-A- 0345299 | 89-12-13 |